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(54) Drug delivery system

(57) Pharmaceutical compositions comprise particulate drug carriers encapsulating a pharmaceutically active substance, which are characterized in that a porphyrin is present at the surface of said carriers. Carriers of choice are liposomes or biodegradable microspheres. The compositions can be used for the targeting of various drugs, such as anticancer agents, to the site where such drug is required.

GB 2 146 525 A

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SPECIFICATION

Drug delivery system

The invention relates to a novel drug delivery system, adapted to target and deliver drugs to tumor sites.

The drug delivery system is based on a combination of drugs carried by liposomes, with porphyrins bound to the outermost layer of the liposomes.

Various porphyrin species may be used, and the choice of the most suitable one depends on the various parameters of the drug delivery system.

Porphyrins have been known to accumulate in various types of cancerous tissues, showing preference for malignant versus similar non-malignant ones as well as against many types of normal tissues.

There are five known porphyrin IX species, namely: Hematoporphyrin IX (HP); Hematoporphyrin IX derivative (HPD); Deuteroporphyrin IX (DP); Mesoporphyrin IX (MP), and Protoporphyrin IX (PP).)

The structural formulae of all these are given in Figure 1. All of these are available commercially. Their spectral properties in aqueous and non-aqueous media are well known.

When a drug is administered in vivo in its free form, this can give rise to two types of problems: (1) Interference with the effectiveness of the treatment resulting from drug levels at the target which are too low (or non-existent). (2) Undesirable side-effects brought upon by the presence of the drug at tissues and organs other than the target.

A potential means to overcome, or at least to reduce, problems of drug delivery in its free form, in vivo, would be to "package" the drug in a carrier/delivery system, thus providing protection for both the drug and the environment into which it is introduced.

Several types of drug-delivery systems derived from biological material are under consideration currently, such as Liposomes; biodegradable microspheres, monoclonal antibodies; cells such as erythrocytes.

Considering liposomes vs. antibodies, there exist advantages and disadvantages of specific (i.e. proteins) vs. non-specific (i.e. liposomes) carriers.

Liposomes have the advantage of a broad spectrum of drugs they can carry, in terms of size, solubility (aqueous and nonpolar), chemical type and therapeutic activities. Also, the encapsulation of the drug inside the carrier (liposomes) might offer better mutual drug and environment protection than binding to proteins. On the other hand, antibodies, through their cell and tissue specificities, might do better in terms of direction to the target and access at the target - in vivo. We will return to the issue of targeting shortly.

As to cells, although they have similar advantages to liposomes in terms of drug spectrum, immunological and practical considerations will, in all probability, limit their use to the cell-donor only.

Drawbacks

(a) *Liposomes*:

The major current drawback to the realization of the potential of liposomes as drug-carriers in living systems is the following: Absence of effective means to target the liposomes, in vivo, from the site of administration to the site of drug action.

"Effective means of targeting", meaning not only "homing" towards a desired target, but also ability of the liposomes to pass through or avoid anatomical barriers and the ability to compete with the natural targets of liposomes in vivo - namely liver, spleen and circulating macrophages. The latter is of particular importance in targeting cytotoxic drugs against tumors. If the liposomes end in the macrophages then such drugs will not only be ineffective in terms of treating the tumor - but also harmful in terms of impairing host defense systems against the tumor.

The efforts to develop targeting ability for liposomes are concentrated currently in two directions:

(a) Manipulating physico-chemical properties of the liposomes such as type, size, lipid composition. The effort is especially on controlling rate of clearance from circulation, a factor which affects tissue distribution, but also on factors such as dose size and routes of administration.

(b) Modification of the liposomes by ligands through which these delivery systems will acquire targeting ability. Two types of ligands have been investigated: ligands capable of recognizing receptors on the surfaces of specific cells; included in this group are aggregated immunoglobulins, monoclonal antibodies, glycolipids and glycoproteins. The other type being ligands which will cause the destruction of the liposome, thereby releasing its contents, in response to a change in the environment (pH, temperature, etc.).

Even though several of these methods have been successful in vitro, none have yielded, to date, general and effective means for in-vivo targeting.

Other drug-carrier systems:

Drawbacks similar to those discussed above have been encountered with other types of carriers (of biological material) particulate such as protein or other biodegradable microspheres or linear such as antibodies.

Furthermore, even if the use of monoclonal antibodies might be promising in terms of target recognition - these as well as less specific proteins do not offer a general solution. Each type of target requires not only a specific carrier, but also specific protein for each drug species, while liposomes have considerable versatility in this respect.

This invention relates to targeting drug-delivery systems to tumors, specifically to liposomes and biodegradable microspheres, as the drug carriers. The term "drug" in this context includes the two following types:

(a) Substances (or their metabolites) acting directly as anti-cancer agents;

(b) Substances acting indirectly - by activating host defense systems against the tumors.

Encapsulation (or binding) of such drugs can be within the inner aqueous regions and/or the lipid regions of the liposome according to the solubility of the drug. Such processes have been well documented and many examples can be found in literature.

Thus, the emphasis is not on the properties of the liposome as a drug carrier, but rather on turning this carrier into a system which has the ability to target itself in vivo from the site of administration to the site of drug action; the latter being the tumor or its associated macrophages, depending of the drug being of type (a) or (b), defined above. Targeting ability is conferred upon the liposome by a porphyrin bound, covalently or non-covalently, to the outermost membrane of the pre-formed liposome. The porphyrin serving as the targeting ligand is to be one of the following five species: Protoporphyrin IX, deuteroporphyrin IX, mesoporphyrin IX, hematoporphyrin IX, and hematoporhyrin IX. There exist no restrictions as to liposome type or lipid composition.

There may also be used other developed types of particulate drug carriers such as protein microspheres, and especially albumins, and such based on polydextran microspheres, where porphyrins of the type set out herein are provided in or on the outermost surface so as to target them to specific tumor sites.

General description of the system:

The targeted drug-delivery system contains three elements: the liposome, the encapsulated drug, and the targeting ligand. Each element will be described in detail below.

Liposomes: Liposomes are spherical membrane-enclosed microparticles containing two types of phases - lipid and aqueous. Liposomes range in size from 20 nm to several um diameter. They can be classified according to the number of lamella, giving two types (uni and multi) of according to size, giving three types. Using the latter classification the following types are recognized: SUV-small unilamellar vesicles, obtained usually by sonification. These liposomes are the smallest in terms of diameter and of inner space. MLV-multilamellar vesicles containing 8-15 concentric lipid bilayers interspaced by aqueous phases. MLV are obtained usually by bringing a film of lipid (thinly spread) in contact with the desired aqueous phase under agitation. These liposomes are intermediate in size, especially in terms of inner aqueous space. LUV - large unilamellar vesicles, obtained by several techniques such as reverse phase evaporization, ether or ethanol injection, detergent dialysis or specific methods for a given lipid. These liposomes are the largest, in terms of diameter and of inner aqueous space.

(ii) *Biodegradable microspheres*: Biodegradable microspheres, for example those made of protein (such as albumin, casein) are spherical microparticles of heat denatured and/or cross-linked protein. In difference from liposomes which have an aqueous phase(s) inside, the protein microspheres are protein throughout. Such microspheres range in size from under 1 μm to 300 μm (diameter). Protein microspheres are stable in aqueous phases as well as in organic solvents (i.e. they are not soluble in organic phases). The microspheres are degradable and can be completely degraded by the application of proteolytic enzymes such as chymotrypsin, trypsin or pepsin.

Drug: The two types of drugs are as defined above. Several examples will be cited here: Drugs of type (a) - adriamycin, daunomycin, actinomycin D, methotrexate, ara-C. Drugs of type (b) - Muramyl dipeptide (MDP) and analogues, macrophage activating factors (MAF). In terms of size, drugs ranging from several hundred daltons of giant macromolecules can be encapsulated, though encapsulation of the larger ones is obviously limited to large liposomes (MLV or LUV) or microspheres.

Targeting ligand: Any of the five porphyrin species (listed in the introduction to this section and illustrated in Figure 1) loaded onto the outermost membrane of the liposome will serve as a targeting ligand. Different targeting requirements will dictate which of the five species is the best for a given situation.

As already discussed above, such a porphyrin, in its free form in a tumor-bearing living system will "target" itself to the tumor. Therefore, provided this ability is not lost upon binding to the liposome - the porphyrin will target the whole delivery system to the tumor. Evidence in support of this is given in the following Examples.

Porphyrins fluoresce upon irradiation in the visible range of light. Peak excitation (394 nm); peak emission (610 nm) and quantum yield vary slightly from one porphyrin species to another. For each porphyrin excitation and emission peaks shift to the red, and quantum yield increases upon porphyrin transition from aqueous to organic media.

Thus, the spectral properties of these porphyrins are probes for a qualitative indication whether the porphyrin is in the liposomal membrane and for quantitative determination of the liposome-associated porphyrin.

The porphyrins can be bound to all types of liposomes, with no restriction on lipid composition and to

biodegradable microspheres.

General preparation procedures:

(a) *Liposomes - preparation and drug-encapsulation*:

Liposomes can be prepared from commercially-available natural and synthetic lipids, as well as from lipids extracted from biological sources at the lab, prior to liposome formation. *MLV* (see Ryman et al (1979) in Lyzozomes in App. Biol. and Ther. *6*, (Dingle JT, Jackes PJ and Shaw TH eds), pp.549-574, North-Holland Pub.Co., Amsterdam; Fidler et al, (1980) Cancer Res. 40, 4460. Knight CG, ed.(1982), Liposomes: From Physical Structure to Ther. Apps., Elsevier, Amsterdam; Wilschut J. (1982) in Liposome Methodology in Pharmacology and Cell Biol. (Leserman LD and Barbet J. eds) p.127,131, Inserm, Paris.

The desired lipid mixture dissolved in an organic solvent (usually chloroform), and nitrogen-flushed, is deposited as a thin film in a round-bottomed flask by rotary evaporation.

The desired aqueous solution (swelling solution) is then added and the dispersion of lipid in it is achieved by vigorous mixing (vortex, shaker, agitation, etc.). During this process the reaction mixture should be kept above the phase transistion temperature of the lipids.

Encapsulation: Substances intended for encapsulation in the aqueous compartments of the liposome are included in the swelling solution. Low ionic strength and a trace of Mg^{2+} (in the case of charged macromolecules) increase the efficiency of encapsulation.

Substances to be encapsulated in the lipid regions of the liposome can be added either to the initial "lipid in organic phase" solution or dispersed in the aqueous swelling solution. If it is desired to add such a substance to the outer membrane only, it can be incubated with preformed liposomes, as will be described later for porphyrins.

Separation of the liposome preparation from excess unreacted materials can be achieved either by centrifugation or by gel-filtration.

The conditions of centrifugation required to pellet the preparation depend on the lipid composition. The pellet can be subjected to washings by buffers as well as agents promoting the hydrolysis of drug associated with the liposome and pelleted with it - in contrast to encapsulated drug. For example - proteolytic enzymes for proteins, nucleases for nucleic acids, etc.

Gel-filtration can be performed using sepharose 2B, 4B, 6B, well as sephadex gels, the liposomes usually coming out in the void volume. Removal of associated (in contast to encapsulated) drug can be performed as described above.

The MLV preparation obtained is usually a heterogeneous mixture in terms of liposome size. Filtration on the gel affords choosing a sample with increased size homogeneity compared to the original preparation, but the preparation (liposome suspended in the eluting buffer) is rather dilute. Centrifugation, on the other hand, does nothing to improve size homogeneity, but does yield a concentrated preparation (the pellet) which can be diluted as desired. Thus, each method has its advantages and disadvantages and can be chosen according to need. Further reduction in size heterogeneity can be achieved by passing the preparation through filters with fixed pore sizes.

SUV: This type of liposome is prepared from MLV by sonication. The encapsulated drug, can be added either during the MLV preparation as detailed above, or during the sonication. Conditions of sonication, including time and temperature, depend on the type of sonicator used (probe or bath), on the lipid composition and on the lipid concentration. As to separation of the preparation, gel-filtration or centrifugation can also be used. Centrifugation requires high g values and prolonged periods of time; the SUV remain in the supernatant.

LUV: There exist several procedures for the preparation of such liposomes; one being REV (reverse phase evaporation). A lipid film is deposited similar to that in MLV preparations, on the bottom of a test tube, and is dissolved in diethyl ether or in a similar solvent of low boiling point. An aqueous phase is added, with excess of organic over aqueous phase. After nitrogen flushing the test tube is capped and the system briefly sonicated in a bath-type sonicator. The emulsion is brought to a gel state by rotary evaporation (at two steps of pressure); additional aqueous phase is added, followed by vortexing and additional evaporation (on a rotary evaporation). Encapsulated substances can be added to the initial lipid-film forming solution, according to their solubility properties, to the organic phase or to the first aqueous phase. Separation of the liposomes can be as described for MLV.

(b) *Porphyrin-liposome binding*

The porphyrins are bound to the preformed liposomes. Liposomes of any of the three above types can be used. The porphyrin can be attached to the outermost membrane of the liposome by covalent or physical (i.e. hydrophobic) bonds.

Non-covalent binding. Porphyrins have an affinity for non-polar media; when porphyrin in an aqueous solution is contacted with membrane-enclosed particles, it results in solvation of the porphyrins in the lipid regions of the membrane. Upon incubation of a porphyrin solution with liposomes, a major part of the porphyrin binds to the liposome.

MLV-porphyrin and LUV-porphyrin species can be separated from excess of unreacted and loosely-associated porphyrin by centrifugation or gel-filtration. For SUV-porphyrin only the latter method is applicable. The tightly-bonded porphyrin is not lost by repeated washings. All porphyrin-containing systems must be protected from exposure to light.

Covalent binding: The porphyrin is "anchored" to the outermost membrane by cross-linking at least one of its carboxylic residues to an appropriate amine residue in the liposomal membrane. This can be effected by the cross-linker carbodiimide. The amine supplied to the liposome by the inclusion of such lipids as phosphatidylethanolamine or phosphatidylserine. Separation and purification of the product, as well as determination of the state of the bound porphyrin is as detailed above.

Microspheres - preparation and porphyrin microsphere binding drug encapsulation

Preparation: There are two major procedures for the preparation of protein microspheres (see Zolle et al, Intl. J. Appl. Rad.Isotop. *21*, 155-167 (1970); Scheffel et al. J.Nuc.Med. *13*, 498-503 (1972); Royter et al. J.Parent.Sci.Technol. *37*, 34-37 (1983); Lee et al. Science *213*, 233-235 (1981).Both procedures start from a heterogeneous system in which protein-containing aqueous droplets are dispersed in an excess organic phase. One procedure proceeds to aggregation of the protein (within the droplets) by heat-denaturation and then the organic phase is an oil. In the other procedure the protein is aggregated through cross-linking. The reagent (glutanaldehyde) is pre-included in the droplets, temperature is ambient or below and the organic phase is an ether/oil mixture. Sonification (probe and/or bath), rates of reactants addition, temperature and rate of stirring, can be manipulated to achieve stable protein aggregates having the desired microsphere shape and to have some control on size distribution of the preparation.

Encapsulation: Albumin, the protein of choice for most protein microspheres reported so far, can bind substances by noncovalent interactions (both polar and nonpolar) and by covalent bonds (cross-linking). Thus a wide variety of drugs, in terms of functional groups, can be protein-bound. The drug can be bound to the native protein (or included in the protein-containing aqueous droplets) by any of said types of binding, before the microsphere-formation process is carried out. This results in drug-encapsulation inside the microsphere as well as on its surface. Alternatively, the drug can be bound, by any of the said types of binding, to the surface of preformed microspheres. In this case, if the drug is mall enough, some of it might also diffuse into the interior of the microsphere,.

Following the procedures used with liposomes, the porphyrins can also be bound to the surface of microspheres. Albumin microspheres are especially suitable, since porphyrins are known to have a high affinity for them (noncovalent binding) and can obviously be bound covalently, or cross-linked as described above.

Characterization of the targeted drug-carrier:

The term "targeted drug-carrier" will be used here for the following: Liposomes encapsulating a drug or drug-model and having a porphyrin in its outermost membrane.

Liposomes, and liposome-carrying drugs have been characterized extensively. The targeting device is the core of the present invention.

The following properties will be discussed:

(a) Spectral; (b) Loading capacity; (c) In-vitro stability.

(a) *Spectral properties*: The peak fluorescence emission of a porphyrin in an organic medium, and this includes lipid regions of liposomes, is red-shifted with increase in intensity, compared to aqueous medium (at neutral pH). Data for the five species are given in Table 1.

TABLE 1

Fluorescence Properties of Porphyrins in Aqueous and Membrane Media:

Porphyrin	*Aqueous Medium (Buffered to pH=7.2)*		*Membrane Medium (Liposomes)*	
	Excitation (nm)	Peak Emission (nm)	Excitation (nm)	Peak Emission (nm)
DP	394	610	397	623
HP	394	615	397	625
HPD	394	617	397	627
MP	390	612	397	625
PP	394	623	410	635

(b) *Loading capacity*:

Efficiency of loading is defined as the percentage of bound porphyrin, out of the total of porphyrin in the initial reaction mixture.

Typical data for HPD loaded, non-covalently, onto LUV and MLV of several different lipid compositions are listed in Table 2. The data demonstrate the major characteristics of this process, in terms of loading capacity: The higher the excess of lipid over porphyrin the higher the efficiency, the increase of efficiency being non-linear and saturating. The data also demonstrate that through control of the experimental conditions a desired degree of loading can be obtained.

TABLE 2A

Efficiency of Loading of HPD onto Liposomes of various Types and Compositions: Hydrophobic Association

Liposome Composition	*Type*	*Moles lipid/moles HPD in initial reaction mixture*	*Efficency of loading (%)*
PC	LUV	1.8×10^4	95
		3×10^3	85
		1×10^3	80
	MLV	20	21
		4	12
		2	5
PC/Cerebroside (19/1)	MLV	50	47
		20	47
		4	12
		2	6
PC/PS (7/3)	MLV	50	43
		25	37
		4	20
		3	16
PC/PS (9/1)	MLV	3	8
PC/PE (9/1)	MLV	3	18

TABLE 2B

*Efficiency of Loading HP onto Albumin (BSA) Microspheres, * on covalent association.*

Moles protein/moles HP in initial reaction mixture	*Efficiency of loading (%)*
1.5×10^5	40
7.5×10^4	33
3×10^3	23
1.5×10^3	15
7×10^2	9
3×10^2	6

* Average microsphere diameter : 10μM

(c) *In vitro stability:*

Stability of the preparation, in terms of the fate of the porphyrin in the liposomal membrane, is characterized by the retention of the porphyrin within that membrane with time. This can be assessed by following the fluorescence of the liposome bound porphyrin. Typical data for non-covalently bound PP and HPD, in MLV of several lipid compositions, are listed in Table 3. The preparations were kept at 10°C (i.e. above the phase-transition temperature of the lipids), suspended in phosphate-buffered saline of pH=7.2, and the fluorescence of the intact liposomes recorded at desired intervals. %-retention of the membrane-bound porphyrin was calculated as the ratio of the peak intensity at time=t, to that at time=0, the latter defined as the time immediately after completion of the preparation. %-retention of total porphyrin in the liposome (i.e. membrane and aqueous regions) was calculated as follows:
Aliquots were taken at time=t and time=0, the liposomes dissolved in 0.6 % triton and the porphyrin concentration determined fluorimetrically against a calibration curve. %- retention gives the ratio of total liposomal porphyrin at time=t, to that at time=0.

The data in Table 3 show that there is a considerable retention of porphyrin in membrane regions of the liposome, even after four days. This allows use of the preparation not only as customary, (i.e. within few hours of preparation), but also up to at least four days - from the aspect of stability of the targeting device.

TABLE 3

Retention of Porphyrin in Multilamellas Liposomes

Non-Covalent Binding

Porphyrin	*Lipid Composition*	*Lipid/Porphyrin Concentration ratio in initial reaction mixture*	*% Retention in Liposomes Membrane bound*		*Total*
			24 hours	*96 hours*	*96 hours.*
PP	PC	67	67		
	"	7	86		
HPD	PC:PS	42		100	
	(7/3)	21		90	100
		4		70	100
		2		50	92
	PC:Cerebroside	51		60	
	(19/1)	25		80	
		5		70	
		3		70	

Excitation and peak emission are as listed in table 1.

Furthermore, the loss of the membrane-bound porphyrin does not seem to be by leakage from the liposomal membranes to the aqueous medium in which the liposomes are suspended. As also seen in Table 4, there is almost complete recovery of the porphyrin from the toal liposome, even at 96 hours. Thus, the decrease in the fluorescence signal of membrane-bound porphyrin with time, can be due to the following processes:
Migration of porphyrin from the outermost membrane of the liposome to inner aqueous spaces, the intensity of the fraction migrating from organic to aqueous medium decreasing.

Porphyrin aggregations in the liposome's membrane and the aqueous phases resulting in partial quenching of the fluorescence signal.

Examples:

Example 1

Encapsulation of a dug-model in MLV

The following example demonstrates the encapsulation of a drug-model, rather than specific drugs, and will also serve for in vivo examples to be detailed below. The rationale for a drug-model is the following: As already discussed, the central issue is not encapsulating drugs in liposomes (this has been carried out and reported on extensively), but targeting. Furthermore, until the site to which the porphyrins target the carrier system is determined, at the sub-organ level (i.e. tumor-associated macrophages or tumor cells themselves) attempting to target a specific drug by the means according to the invention, might be misleading. On the other hand, a suitable drug-model, with known behavior in animal models in its free form, to which a radioactive label can be attached, should be useful for studies at all levels.

To this end we have focused presently on bovine serum albumin (BSA) which can be easily iodinated, to give BSA^{125}-I.

Encapsulation of the protein was carried out according to the following procedure: The swelling solution (see section on preparation of MLV) was made of phosphate buffer at pH 7.2 (3.3 mM buffer, to maintain a low ionic strength) with a trace of Mg^{2+} 1 mM) and the desired amount of protein (usually in the range of 50-2000 ug protein per ml, for 3 mg lipid). The liposome-associated protein was pelleted by centrifugation, followed by 3 cycles of suspension in phosphate-buffered saline PBS) and re-centrifugation. Liposomes composed of PC or PC/Cerebroside were centrifuged in a Sorvall centrifuge for 30' at 15000 rpm. Liposomes composed of PC/PS were centrifuged in a Beckman Ultracentrifuge, at 220000 g for 60'. To verify that the associated protein is encapsulated inside the liposome, preparations were subjected to treatment by proteolitic enzymes, the protein determined before and after the treatment. No significant reduction in the liposome-associated protein was detected after the enzymatic treatment.

Quantitative determination of the protein was carried out according to the Lowry method, adapted to membrane enclosed particles by inclusion of detergent to release the protein. For samples containing BSA-I^{125} quantitative determination was carried out in the intact liposomes, by counting the radioactivity.

Typical results for several lipid compositions are listed in Table 4, presented as % BSA encapsulated, out of the total protein in the initial reaction mixture. All samples had the same lipid concentration (total lipid). The data clearly show that the efficiency of encapsulation increases with the increase in excess of lipid over protein. Also the efficiency under similar experimental conditions is higher in the charged (i.e. PC/PS) vs. the neutral (PC only) liposomes. This is in agreement with the established data on the larger size of internal aqueous space in charged vs. neutral liposomes.

TABLE 4

Efficiency of Encapsulation of BSA inside liposomes (MLV), of various lipid compositions

ug lipid/ug Protein in initial reaction mixture	*% BSA encapsulated*	
	PC	*PC/PS* (7/3)
50	42	65
30	28	36
20	15	24
7	8	8
5	5	5
2	1.2	3.3

Example 2

Testing the MLV/BSA-I^{125} preparation, in vivo

The following example tests the tissue distribution of the drug delivery system itself (i.e. without the targeting ligands) in a living system. Tissue localization of the liposomes is assumed to be similar to the experimentally-determined localization of the encapsulated protein. The data, presented in Tables 5 and 6 in which the fate of free and encapsulated protein are compared, supports this assumption since marked differences are observed. If within the time intervals studied, especially up to 3 hours post injection, there was extensive leakage of the protein from the liposomes (and it should be emphasized that these are MLV. SUV behave differently in vivo), then free and encapsulated protein should have been indistinguishable.

The experimental procedure for the data presented in Tables 5 and 6 was the following. MLV composed of PC only and encapsulating BSA-I^{125} were injected into femal mice. To a control group of similar mice free BSA-I^{125} was injected. At several time intervals animals were sacrificed and the radioactivity in blood and several organs was counted. Data are listed as % counts, of the total dose given.

TABLE 5

Localization of BSA^{125}-I at three hours post-injection

Organ	*% Dose (per organ)* free BSA^{125}-I.	*% Dose (per organ)* Liposome-encapsulated BSA^{125}-I.
Blood*	28	6
Liver	5	11
Spleen	.5	1.5
Kidney	1.8	1.0
Lung	1.4	1.3

* For blood the data is per gram.

TABLE 6

Time-Course of Localization of Liposome-Encapsulated BSA-I^{125} in Liver and in Spleen

Time post-injection (hours)	*% Dose (per organ)* Liver	*% Dose (per organ)* Spleen
1	18	2.5
3	11	1.5
6	3.5	.5
24	2.0	.5

Example 4

Porphyrin loading onto liposomes

Several examples for this issue have already been given in previous sections, where the experimental procedures for preparation as well as product characterization of the porphyrin-liposome species have been detailed. Therefore, to avoid redundancy the reader is referred to Tables 1 - 4 and the accompanying text.

Example 5

Targeting in vivo

DMBA-induced carcimona was transplanted s.c. to the left leg of a mouse. MLV, made from PC, containing encapsulated BSA^{125} and loaded with HPD (noncovalently), were injected i.v.

At 3 hours post injection, the animals were sacrificed and the radioactivity in both the tumor-bearing leg and an untreated leg counted. The index of localization, defined by the following equation, was calculated.

$$\text{Index} = \frac{\textit{CPM in tumor-bearing leg (per mg tissue)}}{\text{CPM in untreated leg (per mg tissue)}}$$

For controls, similar mice were injected with protein-encapsulating liposomes lacking HPD or with free protein.

The results of these experiments are summarized in Table 7. In animals bearing tumors of 1-2 cm diameter (first row), the findings show quite clearly that the index of localization is close to 1 (1.31 ± .23) when the protein is administered in free form. Encapsulating the protein inside liposomes, without the addition of HPD, results in an increase of localization (41% over free-form delivery) in the tumor-bearing organ. This is increased to a significant extent (86% increase over free-form delivery) when the full delivery system (i.e. liposomes encapsulating protein and loaded with HPD) is used. This increase represents real and significant

targeting in vivo, from the site of administration to the tumor-bearing organ.

This pattern is repeated with the second experimental system, in mice with smaller-sized tumors (0.2-0.7cm) (second row), although the degrees of increase in localization are smaller. The index of localization is close to 1 in the case of free-form delivery, with an increase of 20% when the partial delivery system (i.e. no HPD) is used and a 33% increase when the complete delivery system is used.

As an additional control, measurements were taken in animals in which a tumor had not developed (third row). In such cases, the treated and untreated legs are similar (non-tumor bearing) and thus the index of localization should be 1 regardless of the composition of the delivery system. The data show that this expectation is indeed fulfilled.

The increase in tumor localization observed in those animals receiving only the partial delivery system might be the result of the phagocytic activities of the tumor-associated macrophages. Therefore, if indeed the increase in tumor localization in the animals receiving the complete delivery system resulted from tumor-affinity conferred upon the liposomes by the porphyrin, while the increased tumor-localization in the case of partial delivery systems is due to the macrophage involvement, then the actual targeting achieved is better than what the data indicate.

TABLE 7

In vivo Targeting of $BSA\text{-}I^{125}$ -Encapsulating Liposomes to DMBA-induced Carcinoma

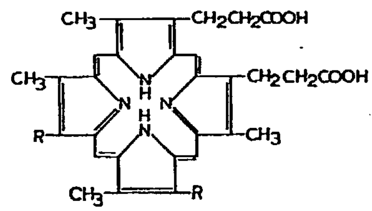
Tumor size (diam,cm)	*Index of localization of $BSA\text{-}I^{125}$*			*Increase in tumor localization of encapsulated over free protein (%)*	
	in free form	*encapsulated in liposomes with no HPD*	*encapsulated in liposomes with HPD*	*- targeting ligand (i.e. HPD)*	*+ targeting ligand (i.e. HPD)*
1-2	1.3 ± .23 (7)	1.85 ± .23 (7)	2.43 ± .58 (7)	41	86
.2-.7	1.23 ± .09 (5)	1.48 ± .19 (4)	1.63 ± .21 (4)	20	33
0 (tumor not developed)	-	1.6	1.02	-	-

(numbers in parenthesis indicate number of animals in group

LIST OF ABBREVIATIONS

BSA	-	Bovine Serum Albumin
$BSA\text{-}I^{125}$	-	Bovine Serum Albumin, Iodinated
DP	-	Deuteroporphyrin IX
HP	-	Hematoporphyrin IX
HPD	-	Hematoporphyrin IX, derivative
LUV	-	Large Unilamellar vesicles
MAF	-	Macrophage activating factors
MDP	-	Muramyl dipeptide
MLV	-	Multilamellar vesicles
MP	-	Mesoporphyrin IX
PC	-	Phosphatidyl Choline
PE	-	Phosphatidyl ethanol amine
PD	-	Protoporphyrin IX
PS	-	Phosphatidyl Serine
SUV	-	Sonicated Unilamellar Vesicles

Figure 1: Formula structures of porphyrins



Porphyrin		*R*
Deutero	(DP)	H
Hemato	(HP)	$CH(OH)CH_3$
Meso	(MP)	C_2H_5
Proto	(PP)	$CH{=}CH_2$

Hematoporphyrin derivative (HPD): A preparation obtained by acetic acid treatment of HP. According to TLC and HPLC analysis there are 4-7 components, the major being HP (55%), HVD (monohydroxyethyl monovinyldeutero porphyrin) (25 %) and PP (5%).

CLAIMS

1. A pharmaceutical composition comprising a particulate drug carrier selected from liposomes and biodegradable microspheres, encapsulating a pharmaceutically active substance, a porphyrin being present on the surface of the outermost layer of the said carrier.
2. A pharmaceutical composition according to claim 1, wherein the liposome is of the SUV type.
3. A pharmaceutical composition according to claim 1, wherein the liposome is of the MLV type.
4. A pharmaceutical composition according to claim 1, wherein the liposome is of the LUV type.
5. A pharmaceutical composition according to any one of claims 1 to 4, wherein the carrier is a protein microsphere.
6. A pharmaceutical composition according to any one of claims 1 to 5, wherein the porphyrin is selected from hematoporphyrin IX (HP), hematoporphyrin IX (HPO), deuteroporphyrin IX (DP), Mesoporphyrin IX (MP), and protoporphyrin IX (PP).
7. A pharmaceutical composition according to any one of claims 1 to 6, wherein the pharmaceutically active substance is an anti-cancer drug selected from adriamycin, daunomycin, actinomycin D, methothrexate, ara-C.
8. A pharmaceutical composition comprising liposomes or biodegradable microspheres containing the active drug and a porphyrin on the outermost surface of the liposomes, substantially as hereinbefore described and with reference to any of the Examples.
9. A method for the production of a pharmaceutical composition according to claim 1, substantially as hereinbefore described and illustrated by the Examples.
10. A pharmaceutical composition when prepared by a method according to claim 9.

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